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NOTIFICATION OF ELECTION

(PCT Rule 61.2)

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Date of mailing (day/month/year)

13 January 1998 (13.01.98)

International application No.

PCT/SE97/01003

Applicant's or agent's file reference

International filing date (day/month/year)

09 June 1997 (09.06.97)

Priority date (day/month/year)

10 June 1996 (10.06.96)

Applicant

SCHRÖDER, Ulf

1. The designated Office is hereby notified of its election made:



in the demand filed with the International Preliminary Examining Authority on:

12 December 1997 (12. 2.97)



in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was

was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

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PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

REC'D 14 OCT 1998

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Applicant's or agent's file reference	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/SE97/01003	International filing date (day/month/year) 09.06.1997	Priority date (day/month/year) 10.06.1996
International Patent Classification (IPC) or national classification and IPC ₆ A 61 K 39/39, A 61 K 47/14		
Applicant Pharmatrix AB et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of <u>4</u> sheets, including this cover sheet. <input type="checkbox"/> This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT). These annexes consist of a total of _____ sheets.
3. This report contains indications relating to the following items: I <input checked="" type="checkbox"/> Basis of the report II <input type="checkbox"/> Priority III <input type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step and industrial applicability IV <input type="checkbox"/> Lack of unity of invention V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability, citations and explanations supporting such statement VI <input type="checkbox"/> Certain documents cited VII <input type="checkbox"/> Certain defects in the international application VIII <input type="checkbox"/> Certain observations on the international application

Date of submission of the demand 12.12.1997	Date of completion of this report 23.09.1998
Name and mailing address of the IPEA/SE Patent- och registreringsverket Box 5055 S-102 42 STOCKHOLM Facsimile No. 08-667 72 88 Telex 17978 PATOREG-S	Authorized officer Carl-Olof Gustafsson Telephone No. 08-782 25 00

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/SE97/01003

I. Basis of the report

1. This report has been drawn on the basis of *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.)*:

☒ the international application as originally filed.

☐ the description, pages _____, as originally filed,
pages _____, filed with the demand,
pages _____, filed with the letter of _____,
pages _____, filed with the letter of _____.

☐ the claims, Nos. _____, as originally filed,
Nos. _____, as amended under Article 19,
Nos. _____, filed with the demand,
Nos. _____, filed with the letter of _____,
Nos. _____, filed with the letter of _____.

☐ the drawings, sheets/fig _____, as originally filed,
sheets/fig _____, filed with the demand
sheets/fig _____, filed with the letter of _____,
sheets/fig _____, filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

☐ the description, pages _____

☐ the claims, Nos. _____

☐ the drawings, sheets/fig _____

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the supplemental Box (Rule 70.2(c)).

4. Additional observations, if necessary:

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/SE97/01003

V. Resoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims		YES
	Claims	<u>1-15</u>	NO
Inventive step (IS)	Claims		YES
	Claims	<u>1-15</u>	NO
Industrial applicability (IA)	Claims	<u>1-15</u>	YES
	Claims		NO

2. Citations and explanations

The present application refers to a pharmaceutical formulation for parenteral or mucosal administration of antigens and/or vaccines comprising a) a monoglyceride and b) a fatty acid and the use of a) or b) as adjuvants/vehicles in pharmaceutical formulations.

It is noted that claim 12 does not state that both components a) and b) are to be present in the adjuvants/vehicles.

The International Search Report revealed 3 documents of particular relevance:

1. EP 544612, see the examples.
2. WO 9306921, see pp 8-11 and 45-47.
3. WO 9522989

From doc. 1, example 7 and table 3, it is evident that the adjuvants are made from corn oil consisting of triglycerides, monoglycerides and free fatty acid. However, the present compositions differ from such adjuvants containing large amounts of triglycerides.

Doc. 2 refers to formulations comprising particles of monoacylglycerides eg. monoolein and fatty acids (p 17-21 and 8-11). The proportions - largely monoglycerides - are in concordance with the adjuvants of the present application. These cubic phase particles can be used as vaccine adjuvants (p 45-47). The claims are considered to lack novelty in view of what is known from this document.

Doc. 3 pertains to vaccine adjuvants (paucilamellar lipid vesicles) comprising a mixture of components, mentioning glycol monoesters and fatty acids (p 3, 1 38-39) and mixtures. The examples involve Brij 52 (a polyoxyethylene ether) and oleic acid.

.../...

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/SE97/01003

Supplemental Box

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: V

The recent development in this field shows that mucosal (eg. intranasal) administration of vaccines can produce a protective immunity if liposomes are used as immunostimulating additive (see eg. Vaccine vol 13, 1995, pp 155-62 not cited in the search report). It is therefore considered to be obvious to a person skilled in the art to use or modify the adjuvants suggested in doc. 2 by adding fatty acid components or monoglyceride components from documents 1 and 3. Unless novelty over doc. 2 can be shown and an inventive step over doc.2 in view of doc. 1 or 3 can be made probable with the aid of unexpected results or otherwise, the claims should involve an inventive step.



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 39/39, 47/14	A1	(11) International Publication Number: WO 97/47320 (43) International Publication Date: 18 December 1997 (18.12.97)
(21) International Application Number: PCT/SE97/01003 (22) International Filing Date: 9 June 1997 (09.06.97) (30) Priority Data: 9602280-1 10 June 1996 (10.06.96) SE (71) Applicant (for all designated States except US): PHARMA- TRIX AB [SE/SE]; Spelmanshögden 14, S-174 50 Sundby- berg (SE). (72) Inventor; and (75) Inventor/Applicant (for US only): SCHRÖDER, Ulf [SE/SE]; Spelmanshögden 14, S-174 50 Sundbyberg (SE).		(81) Designated States: AU, CA, JP, KR, NZ, US, Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: IMMUNSTIMULATING LIPID FORMULATION (57) Abstract A pharmaceutical formulation for parenteral or mucosal administration of antigens and/or vaccines to humans and animals, comprising monoglyceride preparations having at least 80% monoglyceride content and where the acyl group contains from 6 to 24 carbon atoms, together with fatty acids where the number of carbon atoms may be varied between 4 and 22.		

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IMMUNSTIMULATING LIPID FORMULATION

The present invention relates to a novel pharmaceutical formulation for administration of antigens and/or vaccines. The preferred route of administration is via the mucosal membranes, however parenteral administration may also be used. The invention also relates to the use of certain compounds (as defined below) as adjuvants or vehicles in such formulation.

Background

An increasing number of specific antigens from different types of organisms (e.g. tumor cells, bacteria, virus and parasites) has been produced using cloning techniques over the last years. However, these antigens are frequently weak immunogens despite their high specificity.

To obtain good protection after vaccination, immune stimulating systems are needed that can enhance and activate the immune system against these weak antigens. Such immune stimulating systems are called adjuvants.

Adjuvants, presently mainly used in animal experiments, includes a highly heterogeneous group of substances; inorganic substances, oil emulsions, charged polymers, neutral substances or substances from bacteria.

There are presently large efforts in research and development in order to obtain a safe adjuvant with high efficacy to be used in humans. However, today there is presently no general adjuvant for this purpose.

Alum hydroxides and alum phosphates were the first two inorganic substances that were used in humans. The immune response obtained is a result of slow desorption of the precipitated antigen on the surface of the particle. Later it was shown that phagocytosing cells were attracted by these alum salts leading to further enhancement of the immune response. However, these salts are not safe since granuloma formation has been reported (Slater et al, Br.J.Dermatol. (1982) Vol. 107, page. 103-108.). Furthermore, the alum salts can not be used for all antigens since all antigens are not adsorb on the surface.

In 1944 Freund introduced his adjuvant consisting of a mixture of vegetable oil, mineral oil, detergents and killed bacteria. The enhancement obtained was partly due to slow release of the antigen from the oil emulsion. Freund's adjuvant can however not be used in humans due to granuloma formation, induction of auto-immune reactions and the non-biodegradable mineral oil. Furthermore, the effect is difficult to control. The active

substance in Freund's adjuvant has been isolated and its structure determined and shown to be N-acetyl muramyl-L-alaninisoglutamate, often called muramyl-dipeptide (MDP).

The adjuvant effect dependent of the particle size of polymetacrylate and polystyrene particles was examined on mice (Kreuter et al, Vaccine, (1986) vol 4, 125-129) by the use of ovalbumin (adsorbed on the particles) as a model antigen with subsequent assay of the immune response. The size of the particles was varied between 62 and 306 nm. The result was that smaller particles enhanced the immune response better than larger. The smaller particles gave a better effect than 0.2% Al(OH)₃. All preparations elicited a higher response as compared to fluid preparations. Similar experiments where particulate systems with smaller size results in a higher immune response as compared to larger particles are known in the scientific literature.

Almost all systems used today for enhancement of the immune response against antigens are particles or is forming particles together with the antigen. In the book "Vaccine Design - the subunit and adjuvant approach" (Ed: Powell & Newman, Plenum Press, 1995) all known adjuvants are described both regarding their immunological activity as well as regarding their chemical characteristics. As described in the book more than 80% of the adjuvants tested today are particles or polymers that together with the antigens (in most cases proteins) are forming particles. The type of adjuvants that not are forming particles are a group of substances that are acting as immunological signal substances and which under normal conditions consists of the substances that are formed by the immune system as a consequence of the immunological activation after administration of particulate adjuvant systems.

Using particulate systems as adjuvants, the antigens are associated or mixed with or to a matrix which has the characteristics of being slowly biodegradable. Of great importance using such matrix systems are that the matrix does not form toxic metabolites. Choosing from this point of view, the main kind of matrices that can be used are mainly substances originating from a body. With this background there are only a few systems available that fulfils these demands: lactic acid polymers, poly-amino acids (proteins), carbohydrates, lipids and biocompatible polymers with low toxicity. Combinations of these groups of substances originating from a body or combinations of substances originating from a body and biocompatible polymers can also be used. Lipids are the preferred substances since they display structures that make them biodegradable as well as the fact that they are the most important part in all biological membranes.

Lipids are characterized as polar or non-polar. The lipids that are of most importance in the present invention are the polar lipids since they have the capacity to form particulate systems in water. Another way of defining these lipids are as amphiphilic due to their chemical structure with one hydrophobic and one hydrophilic part in the molecule thereby being useable as surface active substances. Examples of main groups of polar lipids are mono-glycerides, fatty acids, phospholipids and glycosphingolipids. These main groups can be further characterized depending on the length of the acyl chain and the degree of saturation of the acyl chain. Since the number of carbon atoms in the acyl chain can be in the range of 6 to 24 and the number of unsaturated bonds can be varied there are an almost infinite number of combinations regarding the chemical composition of the lipid.

Particulate lipid systems can be further divided into the different groups as discussed in the scientific literature such as liposomes, emulsions, cubosomes, cochleates, micelles and the like.

In a number of systems the lipids may spontaneously form, or can be forced to form, stabile systems. However, under certain circumstances other surface active substances has to be introduced in order to achieve stability. Such surface active systems can be of non-lipid character but possess the characteristics of the polar lipids having hydrophobic and hydrophilic parts in their molecular structure.

Another factor that has been shown to be of importance is that lipids exhibit different physical chemical phases, these phases has in different test systems been shown to enhance uptake of biological substances after administration to mucosal membranes.

In the classical immunology and in combination with vaccination against different types of infectious agents e.g. bacteria, virus or parasites the prevailing dogma has been to administrate the vaccine subcutaneously or intramuscularly. However, research has during the last years shown that the body has a very effective immunological system that resides in the mucosa. It has been shown that you can administrate vaccines orally, nasally, rectally and vaginally. In the same way as for the classical immunization it has been shown that by mucosal vaccination there is also a need for enhancement of the immunological response by the addition of adjuvants.

In the same way as within the classical immunology where vaccines (antigens) are administrated parenterally, there is within mucosal immunization a great interest in directing the immunological response towards development of humoral and/or cellular response. If you obtain a humoral response it would be important to direct the response in a way that a certain class of antibodies would be obtained. In order to obtain such a goal,

specific immune stimulating agents can be added to the formulation of antigens and adjuvants.

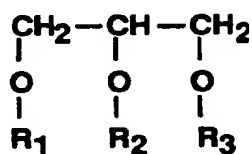
Different types of immune stimulating substances are available. One type is represented by proteins e.g. PHA, Con A, SEA or different types of interferons or interleukines. Another type of substance is represented by MDP, as mentioned above. Additional groups can be characterized as lipid derivatives since they show molecular structures which are amphiphilic. One example of such a substance is called MPL. Another similar substance is Quil A. A number of substances that can be classified within these categories are described in the book "Vaccine Design - the subunit and adjuvant approach" as discussed above.

It would be extremely valuable to be able to make the immunization procedures more effective directing the immunological response towards a certain class or subclass of antibodies and/or to be able to induce a strong T-cell response against the antigens.

Description of the invention

It has now surprisingly been found that parenteral or mucosal administration of a pharmaceutical formulation containing one or two of the following adjuvants with admixed antigens and/or vaccines improves the immune response against the admixed antigens/vaccines. Said pharmaceutical formulation for parenteral or mucosal administration of antigens and/or vaccines to an animal comprise one or more substances selected from

a) monoglycerides of the general formula

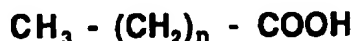


wherein R_1 and R_2 is H and R_3 is one acyl group containing from 6 to 24 carbon atoms, preferably 8 to 20 carbon atoms, even more preferably 14 - 20 carbon atoms and where the acyl chain may contain unsaturated bonds. In a monoglyceride the acyl chain is normally in the R_1 or R_3 position. However there is normally a acyl migration between the 1 and 2 carbons in the glycerol molecule resulting in approximately 90 % is in the R_3 position and 10% in the R_2 position. Thus, in the present invention distilled 1-monoglycerides from Danisco Ingredients (Denmark) with a purity of more than 80 % preferably more than 90 %, more

preferably over 95 % is used. The diglyceride content is maximum 3 % and triglycerides and fatty acid content is less than 1.0 %. The monoglycerides according to the invention normally contains more than more than 80 % of a specific fatty acid, preferably over 90 %.

5 and

b) fatty acids of the general formula



where "n" may be varied between 4 and 22, preferably 8 to 18 and where the acyl chain may contain one or more unsaturated bonds.

10 The formulation according to the invention may comprise additional pharmaceutical excipients selected from the one or several of the following groups; preservatives and osmotic pressure controlling agents, pH-controlling agents, organic solvents, hydrophobic agents, enzyme inhibitors, water absorbing polymers, surfactants and absorption promoters, anti-oxidative agents, and the like.

15 The formulation according to the invention may comprise any antigen and/or vaccine selected among all the antigen and/or vaccines relevant to humans or animals, including marine animals.

This invention discuss lipids which, when mixed with antigens, enhance the immune activity against the antigens thereby functioning as an adjuvant in various vaccine formulations. Especially the invention comprise the use of a formulation for vaccination of the mucosa which can be immunologically activated by nasal, oral, vaginal or rectal administration. The invention also comprise the use of the lipid system for parenteral administration. The use of an adjuvant such as described in the present invention, which can be used both for parenteral as well as for mucosal administration is not limited to humans. Equally important is the use within the veterinary field for the immunization of e.g. cattle, pigs, chickens and the like. Furthermore, there is a large and growing interest in applying both parenteral as well as mucosal vaccines in the field of fish farming. In this area the administration can be performed by incorporation of the formulation in the food. Furthermore, the fish may be allowed to swim for a limited period of time in the vaccine formulation containing the antigens and the adjuvants thus being immunized by the mucosal route via the gills.

In the scientific literature there are reports showing how to enhance the uptake of a biologically active substance after administration to the mucosa together with certain lipids. As an example Li & Mitra (Pharm.Res. vol 13:1, 1996) describes the administration of

insulin mixed with phospholipids in the form of liposomes to the lung. They show that the effect is dependent on the length of the acyl chain and the charge of the particle. Optimal length was 10 carbon atoms and the charge preferably positive. Even negatively charged particles were effective but neutral system were inferior.

5 In the same way de Haan et al (Vaccine, 13:2, 155-62, 1995) describes a mixture of liposomes and the antigen hemeagglutinin. The mixture was administrated nasally to rats whereafter a positive immunological response could be detected. Gupta et al (Vaccine, 14:3, 219-25, 1995) describes that a mixture of diphtheria toxoid together with a non-phospholipid based liposom system administrated parenterally to rabbits results in an
10 immune response which was at the same level as the marketed product which was Alum-adsorbed diphtheria toxoid.

A number of scientific reports also show that good immunological reponses are obtained after administration of liposomes to the mucosa where the antigen is entrapped or adsorbed to liposomes.

15 Studies in vitro on a human cell line obtained from a colon cancer (Caco-2) shows that the best penetrating effect, tested with the model substance mannitol, can be seen with a chain length of 10 carbon atoms. In this case the lipids consisted of the salts of fatty acids. The obtained mixture of these lipids forms together with water micelles (Lindmark et al, J.Pharm.Exp.Ther. 275, 958-65, 1995).

20 Liposomes consists of phospholipids and are formulated by a relatively lengthy and cumbersome process which i.a. involves organic solvents. Furthermore, the phospholipids are expensive.

As described below in the present invention, a similar immunological response can be obtained only by mixing the antigen with a lipid formulation which contains less
25 complicated lipids having a substantially lower price and which can be formulated on a commercial basis in a very simple way.

Another systems that to some extent are similar to the present invention are formulations based on triglycerides. However, these systems are scientifically defined as emulsions of triglycerides where surfactants are used for stabilization. As stabilizers
30 phospholipids or any other type of amphiphilic molecules such as Tween® are normally used. Furthermore, the appearance of such emulsions are normally milky, indicating a size of the oil droplets of about 1 μm . It is well-known for the person skilled in the art that these surfactants are excellent adjuvants. Thus, the adjuvant properties of oil emulsions are primarily due to the characteristics of the surfactant and not of the triglyceride composition.

In PCT/DK94/00062 is disclosed a formulation for the topical administration of antigens and/or vaccines to mammals via the mucosal membranes. Said application disclose in the examples that the only formulation that enhances the immune response is a combination of caprylic/capric acid glycerides with polyoxyethylene sorbitan monoester (Tween 20®).

As exemplified in the present invention it is shown that a combination between a monoglyceride and a fatty acid can stimulate the immune system to produce antibodies and induce protective immunity. Furthermore the present invention shows that the disclosed formulation is able to produce high antibody titers by parenteral administration.

Thus, it was surprisingly found that the administration of antigens and/or vaccines to an animal either via the mucosal route or parenterally using a formulation comprising monoglycerides and/or fatty acids as a particulate lipid system can improve the immunological response towards the admixed antigens and/or vaccines. The monoglycerides are selected from a group with the general formula of 1-acyl-glyceride, wherein the number of carbon in the acyl chain may be varied between 8 and 24, preferably between 12 and 18. The acyl chain may be either saturated or unsaturated. The concentration of the monoglyceride may be in the range of 0.1 - 50 g per 100 ml of water, preferably in the range of 1 - 20 g per 100 ml of water. The fatty acid concentration may be in the range of 0.1 - 50 g per 100 ml formulation, preferably in the range of 1 - 20 g per 100 ml water. When monoglycerides and fatty acids are formulated together the percent ratio of monoglyceride in fatty acid may be varied between 1 to 99 %, preferably between 10 to 90 %.

An enhancement of the immunological response after administration of monoglycerides and/or fatty acids together with antigens and/or vaccines has not been suggested anywhere in the prior art.

The present invention describes that mixtures of antigens with relevant lipids stimulates the body to generate protective immunity. Another advantage of the present invention is the simple formulation process and as compared to entrapment no material (antigen) is lost in the process. As an example can be mentioned that in the process of entrapment in liposomes the recovery is normally 10-20 %. The rest is lost in the process.

Reports in the literature as discussed above, shows that by mixing liposomes and antigen an immune response is detected after administration to the mucosa.

However, the examples in this invention as described below shows that the system can be even more simplified by the use of lipids that are more stable, cheaper and which can be formulated to particles in a more convenient and simplified way.

The invention is exemplified by the following examples showing that the principle of co-administration of antigens, immune stimulating substances associated or in combination with particles function as an adjuvant.

Example 1.

A suspension of mono-olein was produced by adding 3 g mono-olein to 50 ml of a 0.6 % Pluronic-127® solution in phosphate buffered saline pH 7.4, whereafter the mixture was sonicated with a probesonicator for 4 minutes. The obtained milky suspension contained particles with a maximal size of about 2 µm as determined by light microscopy.

Example 2.

A negatively charged micelle suspension of mono-oleate was produced by mixing of 0.5 g of oleic acid with 5 ml of 0.35 M NaOH and sonicated with a probesonicator for 5 seconds. Thereafter 3 g mono-olein and 50 ml 0.9 % NaCl was added whereafter the mixture was probesonicated for 4 minutes. The monester content of the mono-oleate was over 95 % with a acyl chain containing 92 % oleate and 6% linoleic acid. The pH was adjusted to 8.3. The obtained completely clear homogenous solution contained particles with a size of below approximately 0.2 µm as determined by visual inspection. It is known that if a clear solution is obtained the particle size is below approximately 0.2 µm, a slightly opalescent bluish appearance indicated a size of approximately 0.2 - 0.5 µm and if the appearance is milky the size is above approximately 0.8 µm.

Example 3.

A positively charged micelle suspension of mono-olein was produced by mixing 0.5 g lauryl-amine and 3.5 ml of 0.5 M HCl followed by sonication for 5 seconds. Thereafter 3 g mono-olein and 50 ml of water was added whereafter the mixture was probesonicated for 4 minutes. The pH was adjusted to between 4 and 5 using 0.5 M HCl. The obtained completely clear homogenous solution contained particles with a size of below approximately 0.2 µm.

Example 4.

A mixture of particles according to Example 1 and diphtheria toxoid was administrated subcutaneously to mice followed by a booster after 21 days. After 30 days blood samples were obtained which were assayed for IgG antibodies against diphtheria toxin as well as Neutralization titers (NT) using Vero cells. The serum from Alum (n=5) and monoolein (n=5) groups was pooled and assayed. The mice receiving nasal boost and responded (= 3 of 5) were assayed on an individual basis. In arbitrary units is shown in Table 1 the IgG titers and neutralization titers. The results showed that both IgG as well as protective antibody titers were at the same level as compared to the control group which received the marketed product comprising diphtheria toxoid adsorbed on Alum ($\text{Al}(\text{PO}_4)_3$). Also seen is that high IgG titers always were accompanied by high neutralization titers indicating that the formulation does not destroy the antigenic sites that are important for protective immunity.

Table 1.

	Dose diphtheria toxoid μg	IgG titer (arb.units)	NT titer (arb.units)
Alum	15 + 15	32000	40000
Alum	3.5 + 3.5	22000	20000
Mono-olein suspension	15 + 15	24000	20000
Mono-olein suspension	3.5 + 3.5	3500	5000
Nasal boost	7 + 4	45000	10000
Nasal boost	7 + 4	19000	2500
Nasal boost	7 + 4	19500	5000

Example 5.

Particles were prepared according to Example 2 with a final concentration of monoglyceride of 200 mM and of fatty acid of 200 mM. Diphtheria toxoid (2.9 μl , 4.4 mg/ml) was mixed with 200 μl of the micelle suspension and administrated subcutaneously to mice followed by a subcutaneous booster after 21 days. Both the primary and the booster dose of the toxoid was 10 μg . After 30 days blood samples were obtained which were assayed for IgG antibodies against diphtheria toxin. The result showed (Table 2) that the arbitrary IgG titers with respect to the formulation with mono-olein (MO) and oleic acid (C18:1) were at the same level as compared to the control group which received the

present marketed product comprising diphtheria toxoid adsorbed on Alum ($\text{Al}(\text{PO}_4)_3$). The other combinations of monoglycerides and fatty acids gave slightly declining responses which correlated to declining length of the acyl chain (M12 = lauryl-1-glycerate; M10 = capric-1-glycerate; C12 = lauric acid; C10 = capric acid; C8 = caprylic acid). N.D. = Not Done; indicates that there were only five mice in these groups.

Table 2.

IgG response of individual mice (n = 5 or 6) after sc/ sc administration of different formulations containing monoglycerides and fatty acids.

	1	2	3	4	5	6
Alum	18200	18200	9600	9600	18200	N.D.
MO / C18:1	18200	18200	18200	18200	9600	N.D.
MO / C8	9600	9600	4800	9600	4800	9600
M12 / C12	18200	9600	4800	18200	9600	18200
M10 / C10	4800	110	2400	1200	2400	4800

Example 6.

The same procedure as in Example 4 with the difference that the booster dose was given nasally instead of subcutaneously. The dose of diphtheria toxoid was 10 μg both at the primary immunization as well as at the nasal booster administration. In the same experiment a dose-response is demonstrated that is obtained when three different amounts of lipid (see Table 3) was administrated. The arbitrary IgG titer is seen in Table 4. Besides the dose-response effect where lower IgG titers is seen at lower concentrations of lipids there is also seen a higher variability regarding response in the groups receiving lower doses. This variability is not seen at higher dose levels indicating that an adjuvant effect is not only seen with respect to obtaining high titers but also regarding reduction of the variability of the response.

Table 3.

Amount of lipids in μmol administrated to mice sc or nasally.

Dose level	Dose lipid (μmol) sc	Dose lipid (μmol) nasally
high	40	1.5
medium	4	0.15
low	0.4	0.015

Table 4.

IgG titers in individual mice (n = 6) after administration of 2 x 10 µg of diphtheria toxoid to mice either sc/sc or sc/nasally.

	1	2	3	4	5	6
MO / C8 sc/sc high	4800	4800	9600	4800	9600	9600
MO / C8 sc/nas high	9600	1200	4800	4800	4800	9600
MO / C8 sc/sc medium	4800	1200	9600	2400	4800	4800
MO / C8 sc/nas medium	2400	600	2400	600	2400	4800
MO / C8 sc/sc low	300	2400	9600	2400	4800	2400
MO / C8 sc/nas low	600	600	1200	150	4800	150

5

Example 7.

Two different lipid formulation containing mainly medium length acyl chains (Composition A) and long acyl chains (Composition B) were tested. The compositions are seen in Table 5.

10

Table 5.

	Monoglyceride	Fatty acid
Composition A	Monooleate 25 mM Monomyristate 25 mM Monolaurate 25 mM Monocaprinate 25 mM	Caprylic acid 90 mM
Composition B	Monooleate 200 mM	Oleic acid 200 mM

The formulations were administrated to mice s.c. or nasally with a booster after three weeks s.c. or nasally. Blood samples were taken after another week. The arbitrary IgG titers are seen in Table 6.

15

The results in Table 6 demonstrates that in order to achieve a good response after primary as well as booster administration by the nasal route Compositions B is to be preferred.

20

Table 6.

	1	2	3	4	5	6
Composition A sc/nas	2400	4800	4800	18200	4800	4800
Composition A nas/nas	< 100	36400	< 100	< 100	300	< 100
Composition B sc/nas	18200	18200	36400	18200	< 100	N.D.
Composition B nas/nas	4800	9600	18200	18200	2400	9600

Example 7.

- 5 A mixture of mono-olein (200 mM) and caprylic acid (200 mM) was mixed with formalin inactivated influenza virus (strain SDA/94) and administrated s.c. at the first occasion to mice followed by a nasal booster three weeks later. The dose was 0.05 µg HA and blood sample were taken 3 weeks after the booster dose and assayed for agglutination titers (HI) against HA. The results (Table 7) showed that the HI titers in the group receiving the virus together with the adjuvants was at a higher level as compared to the group receiving the virus in PBS.

Table 7.

15 HI titers in mice receiving formalin inactivated influenza virus after s.c. primary injection and nasal booster.

	1	2	3	4	5	6
PBS	N.D.	80	N.D.	40	N.D.	80
MO/C8	320	320	640	160	320	*

N.D. = not detected

* = dead

Example 8.

- 20 Micelles according to Example 2 was mixed with formalin killed rota virus particles and subsequently administrated to female mice. After three immunizations the mice were made pregnant whereafter the new-born mice were challenged nasally with live rota virus. The figures indicate the animals that acquired protection after challenge as compared to the total number of animals in that group. The result from this challenge is seen in Table 8.

Table 8.

Protection after challenge of rota virus to baby mice where the mother was vaccinated with a lipid formulation according to the invention.

Group	Administration	Protection
Saline	im/im/im	2/8
Micelles	im/im/im	4/4
Micelles	im/nas/nas	6/7

- 5 As can be seen from the results there is a good protection both after three intramuscular administrations as well as after a primary intramuscular immunization followed by two nasal administrations.

Example 9.

- 10 To evaluate the toxicity of the lipid formulations these were administrated into the rat nasal cavity whereafter the rats were killed and the nasal mucosa were prepared for light, fluorescence as well as scanning electron microscopy (SEM). Formulations according to Example 1 and Example 2 were tested. Only the mono-olein/pluronic suspension showed minor changes in the mucosal surface using the SEM. No effects could be detected under
15 light or fluorescence microscopy. The micelles containing mono-olein and oleic acid were unable to provoke any changes in the mucosal membranes.

Example 10.

- 20 Caco-2 cells, which are a human cell line originating from a colon cancer can be made to grow as a epithelial mono layer. These cells are frequently used to examine different substances ability to influence the transport of biological substances through epithelial cells and has in a number of experimental systems been shown to give a good correlation to in vivo data regarding uptake from the gut into the bloodstream. As marker substances for transport through the cells Na-flouresceine or mannitol is used. The experiments with the
25 lipid formulations according to this invention showed an enhanced transport through the Caco-2 cells at non-toxic concentrations.

Patent Claims:

- 5 1. A pharmaceutical formulation for parenteral or mucosal administration of antigens and/or vaccines to an animal, **characterized by** comprising one or more substances selected from

- a) monoglyceride preparations having at least 80 % monoglyceride content and having the general formula



15 wherein R₁ and R₂ is H and R₃ is one acyl group containing from 6 to 24 carbon atoms, and where the acyl chains may contain one or more unsaturated bonds

and

- b) fatty acids of the general formula



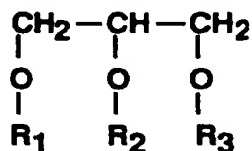
where "n" may be varied between 4 and 22, and where the acyl chain may contain one or more unsaturated bonds.

- 25 2. A pharmaceutical formulation according to Claim 1, **characterized by** having a monoglyceride preparation content of at least 90 %, preferably at least 95 %.
3. A pharmaceutical formulation according to Claim 1, **wherein** the acyl chains of the monoglyceride preparations contains 8 to 20 carbon atom, preferably 14 to 20 carbon atoms and where the acyl chains may contain one or more unsaturated bonds.
- 30 4. A pharmaceutical formulation according to Claim 1, **wherein** the acyl chains of the fatty acid contains 8 to 20 carbon atom, preferably 14 to 20 carbon atoms and where the acyl chains may contain one or more unsaturated bonds.

5. A pharmaceutical formulation according to Claim 1, **wherein** the antigen comprises an antigen and/or vaccine that is selected among the antigen and/or vaccines relevant to humans or animals, including marine animals.
- 5 6. A pharmaceutical formulation according to Claim 1, **wherein** the formulation comprises additional pharmaceutical excipients selected from the one or several of the following groups; preservatives and osmotic pressure controlling agents, pH-controlling agents, organic solvents, hydrophobic agents, enzyme inhibitors, water absorbing polymers, surfactants and absorption promoters, anti-oxidative agents, and
10 the like.
7. A pharmaceutical formulation according to Claim 1, **wherein** the formulation comprises additional adjuvants.
- 15 8. A pharmaceutical formulation according to Claim 1 - 7, **wherein** the formulation is in a form suitable for parenteral or mucosal administration.
9. A pharmaceutical formulation according to Claim 8, **wherein** the formulation is in a form suitable for administration to the mucosa of the nose, mouth, vagina, rectum or
20 the intestine.
10. A pharmaceutical formulation according to Claim 8, , **wherein** the formulation is in a form suitable for administration to the mucosa of the nose
- 25 11. A vaccine or antigen formulation, **characterized by** that 100 g of the final formulation contains:
from 0.01 to 90 g of the antigen/vaccine component
from 0.1 to 90 g of the monoglyceride
from 0.1 to 90 g of the fatty acid
30 from 0.01 to 99 g of water
from 0.01 to 99 g of PBS/saline
and optionally one or more adjuvant and/or excipient.

12. The use of compounds selected from

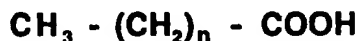
- a) monoglyceride preparations having at least 80 % monoglyceride content and having the general formula



wherein R_1 and R_2 is H and R_3 is one acyl group containing from 6 to 24 carbon atoms, and where the acyl chains may contain one or more unsaturated bonds

and

- b) fatty acids of the general formula



where "n" may be varied between 4 and 22, and where the acyl chain may contain one or more unsaturated bonds

in an amount of 0.01 to 15 g/100 ml of total volume of the formulation as adjuvants / vehicles in pharmaceutical formulations for parenteral or mucosal administration of antigens and/or vaccines to humans or animals, including marine animals.

13. The use of compounds according to Claim 12, **characterized by** having a monoglyceride preparation content of at least 90 %, preferably at least 95 %.

14. The use of compounds according to Claim 12, **wherein** the acyl chains of the monoglyceride preparations contains 8 to 20 carbon atom, preferably 14 to 20 carbon atoms and where the acyl chains may contain one or more unsaturated bonds.

15. The use of compounds according to Claim 12, **wherein** the acyl chains of the fatty acid contains 8 to 20 carbon atom, preferably 14 to 20 carbon atoms and where the acyl chains may contain one or more unsaturated bonds.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 97/01003

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 39/39, A61K 47/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0544612 A2 (THE NISSHIN OIL MILLS, LTD), 2 June 1993 (02.06.93), see examples --	1-15
X	WO 9306921 A1 (GS BIOCHEM AB), 15 April 1993 (15.04.93), page 8 - page 11; page 45 - page 47, claims --	1-15
A	Pharmaceutical Research, Volume 7, No 2, 1990, Parkpoom Tengamnuay et al, "Bile Salt-Fatty Acid Mixed Micelles as Nasal Absorption Promoters of Peptides. I. Effects of Ionic Strength, Adjuvant Composition, and Lipid Structure on the Nasal Absorption of (D-Arg2) Kyotorphin", page 127, see page 131, left column and figure 6 --	1,3,4,6,8-10

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

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"&" document member of the same patent family

Date of the actual completion of the international search

8 October 1997

Date of mailing of the international search report

09.10.1997

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 97/01003

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	US 4073743 A (MICHAEL MIDLER, JR. ET AL), 14 February 1978 (14.02.78) --	1,11
X	WO 9522989 A1 (MICRO VESICULAR SYSTEMS, INC.), 31 August 1995 (31.08.95) --	1,11
T	Vaccine, Volume 14, No 3, 1996, Rajesh K. Gupta et al, "Adjuvant properties of non-phospholipid liposomes (Novasomes) in experimental animals for human vaccine antigens" page 219 - page 225 --	1,11
A	US 5256641 A (MILTON B. YATVIN ET AL), 26 October 1993 (26.10.93), see claims -- -----	1

INTERNATIONAL SEARCH REPORT
Information on patent family members

01/09/97

International application No.

PCT/SE 97/01003

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WO 9306921 A1	15/04/93	AU 2699892 A BR 9206593 A CA 2120359 A EP 0643620 A FI 941538 A JP 7502197 T NO 941191 A US 5531925 A	03/05/93 28/11/95 15/04/93 22/03/95 31/05/94 09/03/95 01/06/94 02/07/96
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WO 9522989 A1	31/08/95	AU 1566095 A CA 2183435 A EP 0746338 A	11/09/95 31/08/95 11/12/96
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